CROSS-IDIOTYPIC SPECIFICITY AMONG MONOCLONAL IgM PROTEINS WITH ANTI-γ-GLOBULIN ACTIVITY*

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In previous studies (1) it was demonstrated that idiotypic antisera to single cold agglutinins showed "cross-specificity" or sharing of antigens exclusively with other proteins which had similar combining activity toward blood group related substances. Other immunoglobulins lacking these specificities failed to precipitate with these antisera. Some evidence was obtained indicating that the antigens detected in this cross-specificity related to the antibody combining sites since they were blocked when the cold agglutinins were bound to the I antigens on the red blood cell.

The present studies were undertaken in an attempt to demonstrate similar cross-specificity between monoclonal IgM proteins with anti-γ-globulin activity through the use of idiotypic antisera. Initial studies indicated that agar precipitation analysis similar to that used for the cold agglutinins presented difficulties primarily because of the reactivity of many of the IgM proteins with the γ-globulin in the rabbit antisera. However Franklin and Frangione did obtain evidence for specific common antigens among the IgM anti-γ-globulins using a precipitation assay (2). In an effort to circumvent our earlier difficulties, studies were carried out utilizing hemagglutination systems. Clear-cut results were obtained and patterns of cross-specificity very similar to those among the IgM cold agglutinins were seen. A preliminary report of these findings has been published (3).

Materials and Methods

The anti-γ-globulins utilized in this study were primarily obtained from individuals with the so-called "mixed cryoglobulin" syndrome. Immune complex types of kidney disease frequently lead to serum analyses and the finding of monoclonal IgM proteins. Most of these proteins were initially purified as cryoglobulins and then separated over Sepharose columns at acid pH as described previously (4). A number of proteins did not form cryoglobulins. These were initially purified by Pevikon block electrophoresis and then isolated from similar Sepharose columns. All of the proteins precipitated with aggregated fraction (Fr) II γ-globulin and this was the primary method of initial screening and criterion of inclusion in the study. In

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addition, agglutination reactions were obtained with latex and other particles coated with γ-globulin. The detailed description of these proteins will be given in a separate study.

The reactivity of each of the anti-γ-globulins with rabbit γ-globulin was determined by their ability to precipitate soluble complexes composed of allotype b4 rabbit γ-globulin-anti-b4 . These were formed at approximately fivefold antigen excess and were completely soluble at 4°C in the presence of IgM proteins lacking anti-γ-globulin activity. In some experiments the b9 rabbit allotype system was employed. The authors are indebted to Doctors T. Kindt and C. Todd for these allotype antisera.

Hemagglutination and hemagglutination inhibition experiments were carried out as described previously from this laboratory (5, 6). The IgM proteins were coated onto human red cells by the bisdiazotized benzidine method (BDB).

Antisera were made in rabbits employing the isolated anti-γ-globulins in complete Freund's adjuvant. These antisera were usually absorbed with 0.2 ml of normal human serum, 2 mg of Fr 11, and 2 mg each of a kappa and lambda IgM protein without anti-γ-globulin activity per milliliter of antiserum.

RESULTS

Precipitation Systems.—Considerable efforts were directed to obtaining a precipitation system for the demonstration of cross-specificity among the monoclonal IgM anti-γ-globulins. 11 different antisera made to 8 of the isolated proteins were studied. It became apparent that agar plate analyses of these proteins were extremely difficult because of their anti-γ-globulin property. Any complexes in the absorbed antisera precipitated in the agar system with the anti-γ-globulins even at relatively high temperatures. The antisera were absorbed with solid absorbents in an attempt to avoid this problem; however, false positive reactions were still observed. Most of the anti-γ-globulins employed in this study showed variable degrees of reaction with rabbit γ-globulin and this was the primary cause of difficulty when rabbit antisera were employed. Table I shows this reactivity in a system where a constant amount of each anti-γ-

TABLE I

Precipitation of Soluble Rabbit γ-Globulin Complexes by Five of Seven Isolated Monoclonal Anti-γ-Globulins

	Protein in pptn
	μg
Ga*	6
Sz*	3
Ea	5
La	6
Wa	28
Ma	32
Bl	43
Po	64
Si	48

System: 50 μg IgM protein + 25 μg b4-anti-b4 complexes.

* Control macroglobulins.

globulin was incubated overnight at 4°C with soluble antigen-antibody complexes consisting entirely of rabbit γ-globulin (allotype-antiallotype). The amount of protein found in the precipitate was used to estimate the reactivity with the rabbit γ-globulin complexes. Control macroglobulins without anti-γ-globulin activity had no effect in precipitating the complexes. All the proteins except Ea and La showed some effect. Some of the proteins caused precipitation at 37°C while others did not.

The reactions of the anti-γ-globulins with rabbit γ-globulin in the absorbed antisera not only gave false positive reactions but it also accentuated the amount of precipitate formed by weak reactions in the cross-specificity analyses. Since this accentuation did not occur with the proteins which failed to react with the rabbit γ-globulin, a false impression of the degree of antigenic specificity was obtained. Some success was finally obtained in precipitation systems by utilizing only the proteins Ea and La mentioned above and then inhibiting their precipitation with F(ab) fragments from the other IgM anti-γ-globulins.

Hemagglutination and Hemagglutination Inhibition Assays.—Considerably greater success was achieved in demonstrating various types of cross-specificity in the hemagglutination system. Table II shows the agglutination titers of red cells coated with various IgM proteins with the absorbed antiserum to Ma anti-γ-globulin. As is evident strong agglutination was obtained with the Ma coat and also the heterologous anti-γ-globulin coats, Wa and Bl. Little or no agglutination was obtained with coats of the six control IgM proteins nor with two IgM anti-γ-globulins, Po and La. Four different absorbed antisera made to proteins Ma and Wa as well as two other antisera to other anti-γ-globulins showed very similar findings with a similar pattern of agglutination. Control antisera made to IgM proteins without anti-γ-globulin activity showed reactivity only with the coat protein used for immunization after similar absorption. Similar results were obtained in these experiments irrespective of whether solid absorbents or soluble proteins were used for the absorption.

TABLE II

Agglutination of Red Cells Coated with Various IgM Proteins at Dilutions of the Absorbed Antiserum to Anti-γ-Globulin Ma

IgM coat	Dilution of antiserum			IgM coat	Dilution of antiserum			
	1/10	1/40	1/160		1/10	1/40	1/160	1/640
Da*	1	0	0	Ma‡	3	3	3	2
St*	tr	0	0	Wa‡	3	3	2	1
Sz*	0	0	0	Bl‡	3	3	2	1
Co*	1	0	0	Ea‡	2	2	1	0
Ch*	0	0	0	Po‡	tr	0	0	0
Mc*	0	0	0	La‡	0	0	0	0

* IgM proteins without activity.

‡ IgM anti-γ-globulins.

Table III illustrates the results of hemagglutination inhibition experiments using the system, protein Wa as the coat and absorbed anti-Ma as the agglutinating antiserum. The same proteins that would serve as coats to give agglutination with this antiserum also showed inhibition of agglutination. These results were clear-cut and an identical inhibition pattern was obtained with the two antisera to Ma. In addition the two antisera to Wa also were inhibited in the same fashion when Ma was used as the coat protein and when additional absorption was made with the negative proteins, La and Po. A total of 14 isolated IgM anti-γ-globulins were tested and 8 showed this exact same pattern of

TABLE III

Cross-Specificity of Isolated IgM Anti-γ-Globulins. Four of the Six Anti-γ-Globulins Inhibit the Reaction While Other Macroglobulins Do Not (Wa System)

	Inhibitor protein concn (mg/ml)						
	1.0	0.25	0.06	0.015	0.004	0.001	0.0002
Ma*	0	0	0	0	0	0	1
Wa*	0	0	0	0	0	tr	2
Bl*	0	0	0	0	0	2	2
Si*	0	0	0	0	0	1	2
Ea*	0	0	0	0	0	tr	2
La*	tr	2	2	2	2	2	2
Po*	1	2	2	2	2	2	2
Ga‡	2	2	2	2	2	2	2
St‡	tr	1	2	2	2	2	2
Sz‡	2	2	2	2	2	2	2
Fr II	0	0	tr	2	2	2	2

Antiserum made against anti-γ-globulin Ma; red cell coat, Wa.

* IgM anti-γ-globulins.

‡ IgM proteins without activity.

inhibition with the different coats and antisera. In each case the degree of inhibition was very similar and contrasted with the very low or absent inhibitions of control proteins. In addition, the same group of anti-γ-globulins were always negative. The positive proteins in these systems were classified as belonging to the Wa group.

Antisera were produced against two of the anti-γ-globulins which were negative in the above systems. Table IV shows the results with the anti-La antiserum which clearly showed a different specificity with La, Po, and Ka being the primary inhibitors. All the proteins that were positive in the above experiments were entirely negative here; only the results for proteins Ma, Wa, and Bl are shown but similar findings were made on the other four proteins of the Wa group. This pattern was a consistent finding with different coats as well as antisera and these positive proteins were classified as belonging to the Po group.

Further detailed studies particularly with the proteins that did not fit into

TABLE IV

Cross-Specificity among Anti-γ-Globulins Which Were Negative in the Wa System

	Inhibitor protein concn (mg/ml)						
	1.0	0.25	0.06	0.015	0.004	0.001	0.0002
La	0	0	0	0	0	0	2
Po	0	0	0	0	0	0	2
Ka	0	0	0	0	2	2	2
Be	0	0	2	2	2	2	2
Ma*	tr	2	2	2	2	2	2
Wa*	0	2	2	2	2	2	2
Bl*	tr	2	2	2	2	2	2
Fr II	0	0	tr	2	2	2	2

Red cell coat, Po; antiserum anti-La absorbed.

* IgM anti-γ-globulins positive in Wa system.

either of the above groups indicated that a considerable number of additional antigens were involved. The unclassified proteins appeared to represent a more heterogeneous group. Protein Be, for example, which is shown in Table IV was clearly not in the Wa group but inhibited slightly in the Po system. However, through the use of other coats and antisera it could be differentiated clearly from the Po group although in a number of systems antigenic similarities were noted.

Preparations of F(ab) and $F(ab')_2$ fragments of two of the anti-γ-globulins showed inhibitory activities and specificity very similar to that of the parent proteins. Isolated light chains from two of the proteins showed no inhibitory effects while the isolated heavy chains showed weak inhibition. All but one of the proteins employed in the study were of the kappa light chain type. The one lambda protein kindly supplied by Dr. Thomas Waldman could not be classified although no antisera were prepared against this protein. Hybridization experiments were not attempted.

Relationship to Antigens in Pooled γ-Globulin and in Other Antibodies.—Fr II γ-globulin and the γ-globulin isolated from single normal individuals inhibited each of the systems described above to a slight degree (Tables III and IV). The degree of inhibition usually required approximately 50 times as much protein as did the positive proteins in both the Wa and the Po groups. Similar results were obtained with normal IgM isolated from a single normal serum. In those precipitation systems where satisfactory results were obtained these normal heterogeneous pools of immunoglobulins also inhibited the positive protein precipitation. However, they failed to precipitate with the absorbed antisera. To a certain degree the inhibition depended on the extent of absorption of the idiotypic antiserum and large amounts of heterogeneous immunoglobulin eliminated the cross-specificity reaction. In some experiments in the hemagglutination inhibition system as much as 20 mg Fr II/ml of antiserum were used for absorption

without eliminating the specificity for the Wa group. Similar results were obtained with F(ab) fragments prepared from the heterogeneous immunoglobulins.

It was apparent from the above experiments that there were a few molecules in these pooled γ-globulins that showed similar antigens to those involved in the cross-specificity groups. This was also brought out in systems employing other antibodies that were known to be very heterogeneous. It was demonstrated some years ago (7) that idiotypic specificities could be brought out in systems where anti-Rh antibodies are used to coat cells and are agglutinated by the idiotypic antisera. The same phenomenon was observed for the cross-specificity reactions. 10 different anti-Rh antisera were analyzed as coats and 2 of them were agglutinated by the absorbed antisera employed above for delineating the anti-γ-globulin groups. This agglutination was found to be specifically inhibited by the same proteins that had been positive with these antisera when anti-γ-globulins were used as red cell coats. Table V shows the results of one of these experiments. The anti-Ma antiserum, which had been used for delineating the Wa group of anti-γ-globulins, agglutinated anti-Rh coat Ri. This was specifically inhibited by Wa group proteins but not by proteins of the Po group or by macroglobulins without activity. The same phenomenon was observed when anti-La was employed with anti-Rh coat Ri; only proteins of the Po group inhibited the reaction and the Wa proteins were entirely negative. Pooled immunoglobulins also inhibited these reactions but, as before, to a considerably less degree than the positive proteins. Isolated anti-Rh antibody Ri inhibited slightly but no more than pooled immunoglobulins. These findings indicated that in the heterogeneous pool of anti-Rh antibodies there were a few antibody molecules with antigens related to those giving the cross-specificity among the anti-γ-globulins.

Relationship to Antibody Combining Sites.—The fact that anti-Rh antibodies, with their combining sites presumably blocked by combination with the Rh sites on the red cells, contained antigens directly related to the cross-specificity

TABLE V

Cross-Specificity Demonstrated in the Agglutination System Employing Red Cells Coated with Anti-Rh Antibodies

	Inhibited protein concn (mg/ml)					
	0.25	0.06	0.015	0.004	0.001	0.0005
Ma*	0	0	0	0	2	2
Wa*	0	0	0	0	2	2
Si*	0	0	0	0	2	2
Po	2	2	2	2	2	2
La	1	2	2	2	2	2
So	2	2	2	2	2	2

Antiserum, anti-Ma absorbed; coat, anti-Rh (Ri).

* Anti-γ-globulins of the Wa group.

reactions of the Wa and Po groups suggested that the antibody combining sites were not directly involved. However, further studies with F(ab) fragments indicated that just the opposite was the case. F(ab), F(ab′), and $F(ab')_2$ fragments of anti-Rh antibody Ri were produced. Each of these bound to Rh positive red cells which were then agglutinated by anti-F(ab) antisera to a similar degree. Similar findings were obtained with anti-light chain antisera. However, when the idiotypic antiserum was used as the agglutinator, completely negative results were obtained for the F(ab) and F(ab′) coats while the $F(ab')_2$ coat gave strong agglutination to titers of $\frac{1}{256}$. It thus appeared that the univalent fragments when attached to the red cell lacked the cross-specificity antigens. The divalent fragments and the whole antibody probably had one of their two sites unbound to the red cell and thus available for reaction with the cross-idiotypic antiserum. It is well known that the Rh sites on the red cell are separated by considerable distances (8) and only one site per divalent antibody would be able to attach to the cell.

DISCUSSION

The hemagglutination and hemagglutination inhibition systems proved very useful techniques for bringing out "cross-idiotypic specificity" between monoclonal anti-γ-globulins isolated from the sera of unrelated individuals. In these tests it was necessary to use as the red cell coat a different anti-γ-globulin than the one used to produce the idiotypic antiserum. After heavy absorption of these antisera with IgM proteins lacking anti-γ-globulin activity along with pooled γ-globulin, these antisera still agglutinated red cells coated with anti-γ-globulins but not those coated with other IgM proteins. It was possible to classify the anti-γ-globulins into two main groups employing these procedures based on antigens common to anti-γ-globulins and not possessed by IgM proteins lacking this activity. The major Wa group appeared quite homogeneous and was brought out with five different antisera. Approximately 60% of the anti-γ-globulins were clearly delineated in the Wa group. The second Po group bore no resemblance to the Wa group being completely negative in the Wa system. However, specific antisera to the Po proteins brought out the Po group in a positive fashion with the Wa proteins being completely negative. Evidence was obtained for additional groups and several proteins were encountered that were negative in both the Wa and the Po systems.

It should be emphasized that each of the absorbed antisera utilized in bringing out these cross-specificities also showed additional antibodies that were completely specific for the proteins used in the immunization. If one employed such a homologous system with agglutination of red cells coated with the immunizing protein, inhibition could only be achieved with immunizing protein. No inhibition was found with any heterologous anti-γ-globulin, nor with Fr II at high concentrations or normal IgM pools. The idiotypic specificity was complete and no similar molecules were found in the six such systems that were used in the

present study. The results were exactly the same as in the case of myeloma proteins studied previously by these techniques (6). Thus each of the anti-γ-globulins, even within the homogeneous Wa group, still could be differentiated from all the others.

Clear evidence was obtained in the present study that the antibody combining site was directly involved in the antigens responsible for the cross-specificity reactions. This was brought out best in the systems employing anti-Rh antibodies as the red cell coats. Certain very heterogeneous anti-Rh antisera were found to contain a few molecules that have antigens closely related to those involved in the cross-specificity reactions and the same delineation of the two major groups could be obtained when this system was used with the same specific antisera. The details of this reaction with idiotypic antisera will be described in a separate publication. Suffice it to say that monovalent F(ab) fragments when attached to the Rh sites on the red cell failed completely to react with these antisera. However, divalent $F(ab')_2$ fragments reacted well and these reactions were inhibited specifically by the anti-γ-globulins of the group known to be recognized by the specific antiserum. Evidence was obtained that because of the known considerable distance between anti-Rh sites on the red cell (8) only one site in a divalent molecule can react while the other is free and available for reaction with the idiotypic antisera. Additional direct evidence for involvement of the antibody combining site was obtained through the use of aggregated human γ-globulin which has a strong affinity for the anti-γ-globulins. Here considerable blockage of the agglutination and of the agglutination inhibition reactions could be demonstrated. In this respect these antigens resembled the idiotypic antigens where Brient and Nisonoff have demonstrated clearly involvement of the antibody combining site (9).

The cross-specificity reactions for the anti-γ-globulins were based on antigens found on anti-γ-globulins but not on other monoclonal proteins lacking this activity (more than 100 control proteins were tested). These antigens, however, were found at very low concentrations in normal immunoglobulin pools. Precipitation experiments were very difficult for the anti-γ-globulins primarily because of the reaction of some of them with the rabbit γ-globulin in the antisera. However, for selected proteins valid results could be obtained which closely paralleled the findings by hemagglutination. Precipitation, however, was not obtained with IgG or the IgM pools although inhibition of precipitation was again apparent. These results suggested that multiple antigens were involved in the anti-γ-globulin specific antigens of the Wa and Po groups and that they appeared together in these proteins while occurring primarily singly at low concentrations in the immunoglobulin pools. It is of interest that antigens similar to these also could be found at low concentrations in very heterogeneous anti-Rh antibodies.

Considerable effort was directed in this study to the determination of the exact fine specificity of the different anti-γ-globulins for the Fc fragment of IgG

proteins of different species and the human IgG subclasses. It has been known for some time that differences can be demonstrated among heterogeneous IgM anti-γ-globulins with regard to specificity for rabbit γ-globulin (10) and with respect to specificity for the IgG3 subclass of IgG (4). Similar findings have been obtained for the monoclonal anti-γ-globulins (11, 12). Considerable differences in the reactivity of the different proteins utilized in this study were observed with antigen-antibody complexes consisting entirely of rabbit γ-globulin (Table I). In this system it appeared that two proteins failed entirely to react with rabbit γ-globulin. One of the unreactive proteins fell in the Wa group and the other in the Po group. With respect to reactivity with different subclasses of human IgG, all proteins reacted with IgG1 proteins but four failed to react with IgG3 proteins. Only one of the latter was in the Wa group and two were included with the three proteins that were not classified in the two major groups. This suggested a possible relationship to the antigenic groupings but no absolute correlation was apparent. It is possible that the proteins that failed to react with the rabbit complexes and the IgG3 proteins actually did so but that their binding affinity was so low that positive results were not obtained in the test systems utilized. Some evidence for this possibility was obtained from the variable degrees of positivity of the proteins that did react.

Another possibility that requires consideration is that no direct relationship to the fine specificity of antigen binding will be obtained even in further studies but that some other broader principle is involved in the cross-idiotypic specificity. The monoclonal cold agglutinins have been known for some years to show a completely parallel type of cross-specificity among each other (1) which is independent of that for the anti-γ-globulins. Here, too, it has proven very difficult to relate the different cold agglutinin specific antigens to fine binding specificity for the blood group related antigens with which they are known to react. Considerable progress has been made recently (13) concerning a number of different types of binding specificities permitting grouping of the cold agglutinins and a more complete answer should be forthcoming. However, both for the cold agglutinins and for the anti-γ-globulins the true antigen, if such a thing exists, is completely unknown and just how these monoclonal IgM proteins arise remains a mystery. If this were known the exact relationships between antigen binding and idiotypic antigens might be more apparent and the extraordinary antigenic similarities of these proteins explainable.

Structural studies have been carried out by Kehoe and Capra on the heavy chains of three of the proteins used in the present antigenic analysis. Proteins Po and La which both belonged in the Po group showed marked sequence similarities in the first (positions 31–37) and second (positions 86–91) major hypervariable regions and contrasted with very different sequences in protein Be which did not belong in either the Wa or Po groups. No information is available concerning how the sequence similarities obtained for Po and La might relate to a peptide previously described as characteristic of certain anti-γ-globulins (2).

It will be of special interest to carry out sequence studies on proteins of the Wa group since they appeared the most homogeneous antigenically. The details of these studies will be published separately.[1] The evidence obtained thus far suggests that sequence similarities in the hypervariable regions of the heavy chains are responsible for the cross-specificity which might be expected in view of the involvement of the antibody combining sites in these antigens. However it was not possible to localize the cross-specificity antigens to either the heavy or light chains of the anti-γ-globulins. Evidence that heavy chain sequences determine the cold agglutinin antigens was obtained in previous studies (1) and this suggests a similar situation for the anti-γ-globulins. It also seems important to ascertain conclusively whether or not these common antigens are limited to single V region subgroups of the heavy chains. The similar proteins, Po and La, belong to the V_HIII subgroup; their light chains are different, V_KIII and V_KI, respectively. Three proteins of the major Wa type have been studied thus far and they all belong to the V_HII subgroup. These preliminary data suggest a relationship.

The work of Braun and Krause (14) and Eichmann and Kindt (15) with homogeneous antibodies to streptococcal antigens in rabbits has demonstrated clearly close idiotypic relationships between the proteins from genetically related rabbits which were not found among outbred animals. Also among inbred strains of mice, idiotypic identity has been found for myeloma proteins of similar combining specificity for the antigen, phosphoryl choline (16, 17). This work has been extended recently by Nisonoff and associates (18) to include different anti-hapten antibodies produced in various inbred mouse strains. In all of this work on closely related animals, the cross-idiotypic specificity was considerably greater than that described above and little or no cross-specificity was found in unrelated animals. However the methodology employed in the present study was quite different and specifically designed for bringing out relationships among proteins from outbred individuals. This was done through the use of an anti-γ-globulin as the red cell coat that was different from the one used to prepare the antiserum utilized in a given experiment, as well as through the employment of relatively limited antiserum absorption. It has not proven possible thus far to obtain monoclonal anti-γ-globulins from related individuals for comparison but this may become feasible through the isolation of anti-γ-globulins that are found at low concentrations in the serum of all normal individuals. It appears probable that within the idiotypic specificities of these proteins two basic elements are present. One of these relates directly to the antibody combining site with similarities in basic structure of the proteins with similar combining specificity obtained from unrelated individuals. The other, probably also involving the combining sites, relates to differences in these proteins from such individuals on a more purely genetic basis and which is reflected in the fact that none of the proteins were idiotypically identical.

[1] Kehoe, J. M., and J. D. Capra. Manuscript in preparation.

SUMMARY

Through the use of absorbed idiotypic antisera prepared against single isolated monoclonal IgM anti-γ-globulins, partial cross-idiotypic specificity was demonstrated with other IgM anti-γ-globulins. Such antisera classified these proteins into at least three groups. The major group which included 60% of the anti-γ-globulins was particularly homogeneous. The anti-γ-globulin specific antigens were detected best in hemagglutination and hemagglutination inhibition systems. They were not found in monoclonal IgM proteins that lacked anti-γ-globulin activity although related antigens were detected at low concentrations in pooled immunoglobulin preparations as well as in heterogeneous anti-Rh antibodies.

Several lines of evidence were obtained indicating that the antibody combining site was involved in the specific determinants. Attempts were made to analyze the fine specificity of each anti-γ-globulin for the Fc fragment of different subclasses of human immunoglobulins as well as those of other species. Differences were observed but these were not readily related to the cross-specificity antigens.

The anti-γ-globulin specific antigens were very analogous to those previously described for monoclonal IgM cold agglutinins. Although each protein could be distinguished from all the others on the basis of individual idiotypic antigens, the antigens common to the specific groups of proteins with each of these activities were prominent and readily detected with multiple antisera. The results indicate basic similarities between proteins of a given activity even in unrelated individuals.

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